Synergistic Effect of Chloroquine and Copper to the Euryhaline Rotifer *Proales Similis*

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Abstract

Chloroquine (CQ) has been widely used for many years against malaria and various viral diseases. Its important use and high potential to being persistent make it of particular concern for ecotoxicological studies. Here, we evaluated the toxicity of CQ alone and in combination with copper (Cu) to the euryhaline rotifer *Proales similis*. All experiments were carried out using chronic toxicity reproductive five-day tests and an application factor (AF) of 0.05, 0.1, 0.3 and 0.5 by multiplying the 24-h LC₅₀ values of CQ (4250 µg/L) and Cu (68 µg/L). The rate of population increase (r , d⁻¹) ranged from 0.50 to 52 (controls); 0.19 to 0.39 (CQ); 0.09 to 0.42 (Cu); and -0.03 to 0.29 (CQ-Cu) and decreased significantly as the concentration of both chemicals in the medium increased. Almost all tested mixtures induced synergistic effects, mainly as the AF increased. We found that the presence of Cu intensifies the vulnerability of organisms to CQ and vice versa. These results stress the potential hazard that these combined chemicals may have on the aquatic systems. This research suggests that *P. similis* is sensitive to CQ as other standardized zooplankton species and may serve as a potential test species in the risk assessment of emerging pollutants in marine environments.

Introduction

Emerging pollutants (EPs) are becoming more common in nature. This is a general term that primarily refers to the compounds that have the potential to enter the environment and are still largely unregulated by existing water quality regulations (Wells et al., 2010). These human-made chemicals include personal care products, pesticides, nanoparticles, microplastic and pharmaceuticals demanded by modern society (Gavrilescu et al., 2015; Egbuna et al., 2021). There are growing concerns regarding the global production of synthetic chemicals. In recent years, 400 million tons per year have been produced, and at least 50% are environmentally harmful chemicals (Gavrilescu et al., 2015). Additionally, human needs could increase EPs' demand in the contemporary world (Bunke et al., 2019).

Pharmaceuticals, including antibiotics, antidiabetic, antiepileptics, antimalarial, analgesics and anti-inflammatories, are frequently reported in freshwater and marine environments (Gavrilescu et al., 2015; Gu and Wang, 2015). Many of these drugs are not biodegraded during the wastewater treatment and are discharged in an active form to aquatic environments (Fang et al., 2012; Liu et al., 2020). Consequently, they are regularly detected in levels ranging from ng/L to mg/L. The bioavailability of pharmaceutical drugs in aquatic ecosystems has a significant ecological effect on the aquatic biota. For instance, in primary consumers such as rotifers and cladocerans, these compounds negatively affect their biological parameters (life span, reproductive rates, generation time, and population increase) (Varano et al., 2017; González-Pérez et al., 2018). On the other hand, pharmaceuticals can alter the behaviour traits of secondary consumers (fishes) and affects their ecological interaction (Brodin et al., 2014). It is documented that pharmaceutical drugs accumulate and biomagnify in the tissues of animals through the food chain (Zenker et al., 2014). Finally, they cause a decrease in the abundance and diversity of aquatic invertebrate communities (Ali et al., 2021). Despite the evident risk, pharmaceuticals are still

necessary to address the current adversities, as exemplified by the COVID-19 (Coronavirus Disease 2019) pandemic (Espejo et al., 2020).

Chloroquine is an antimalarial treatment utilized for more than seven decades, given that it is a safe drug that is easy to obtain at a relatively low cost (Attia et al., 2021). It is a drug repurposing successfully used to treat various diseases in humans comprising VIH, Q fever, influenza H5N1, malaria, hepatitis C, dengue virus, zika virus, and chikungunya virus. Furthermore, it has been tested in cancer patients with promising results (Yan et al., 2013; Plantone and Koudriavtseva, 2018). From this perspective, CQ is considered a drug with current and future potential applications. Chloroquine was one of the first drugs suggested for treating coronavirus disease 2019 (COVID-19) (Colson et al., 2020). Despite limited evidence, it was very early (March 2019) announced as a promising drug candidate against COVID-19; this led to a huge demand for and substantial use of this drug (Roustit et al., 2020). Later (June 2020), it was suspended due to their safety concerns reported by researchers (WHO, 2020).

In the COVID-19 scenario, a CQ dose of 500 to 1000 mg/day for seven days was recommended (Pastick et al., 2020). For other diseases such as malaria and chikungunya virus, CQ is administered in 10 – 25 mg/kg and 250 mg daily, respectively (Taylor and White, 2004; Delogu and de Lamballerie, 2011). Approximately 50% of those amounts are excreted unchanged in the urine and feces; hence, substantial amounts of these wastes enter the aquatic environment due to inadequate treatment of residual waters (Kuroda et al., 2021). Chloroquine has a long half-life in both the human body and the environment (Olatunde et al., 2014; Kuroda et al., 2021). Because of their potential to be a persistent pollutant in water, some mechanisms for CQ degradation have been proposed, but at present, they are not implemented worldwide (Midassi et al., 2020).

Copper (Cu) is ubiquitous in the environment and are thus frequently in marine environments. The US Environmental Protection Agency has listed it as one of the priority contaminants. Cu bioavailability in the aquatic environment may be natural or influenced by anthropogenic (mining, metallurgic industry, aquaculture, agriculture, etc.) sources (Páez-Osuna and Osuna-Martínez, 2015). Cu is an essential trace element for many physiological functions (e.g., mitochondrial respiration, normal cell growth and development and antioxidant defence). However, it can be harmful to human health and aquatic life at high concentrations (Ansari et al., 2003). There is growing concerned about the toxicity of copper to aquatic invertebrates, including marine zooplankton, since this metal's presence in the medium, even at low concentrations (10 – 50 µg/L), adversely affects the population dynamics, which translates into potential risks to the trophic structure of aquatic ecosystems (Schuler et al., 2008; Kwok et al., 2008; Bao et al., 2013; Rebolledo et al., 2021). In some cases, this risk intensifies when Cu is combined with other toxic substances (Bao et al., 2014; Jia et al., 2020).

Rotifers are fundamental in the ecological structure of aquatic ecosystems. These organisms respond quickly to environmental stresses caused by heavy metals, pesticides, pharmaceuticals, among others; hence their relevance in ecotoxicological studies (Snell and Marcial, 2017). The most used endpoints to estimate ecological risk for rotifer populations are acute (LC₅₀) and chronic (instantaneous growth rate

(*r*) toxicity tests (Rico-Martínez et al., 2013; González-Pérez et al., 2018). The *r* is a good measure of response to toxicants since it integrates potentially complex interactions among life-history traits, including reproductive and mortality rates (Forbes and Calow, 1999), which is unattainable through acute tests.

Many aquatic organisms live in environments polluted by a cocktail of toxic substances, including heavy metals and pharmaceuticals. Despite this general assumption, the mixture toxicity of heavy metals and drugs is examined separately in most cases (Watanabe et al., 2015; Lynch et al., 2015). Few studies have examined the toxicity of mixtures like the ones mentioned above (Almeida et al., 2018; Jia et al., 2020). Taking on account (1) the current relevance of CQ as an emerging pollutant, (2) widespread Cu contamination in marine and coastal environment and (3) that in a real scenario, environmental pollutants typically occur as mixtures rather than as individual pollutants (Cedergreen 2014), this work aimed to assess the toxicity of CQ alone and in combination with Cu to the euryhaline rotifer *Proales similis*. The potential of this species as a reliable model organism for marine ecotoxicological studies has been previously demonstrated by Rebolledo et al. (2018), Snell et al. (2019) and Kim et al. (2021).

Materials And Methods

Test species

Proales similis de Beauchamp 1907 (Rotifera: Monogononta) was isolated from a shrimp farm of *Litopenaeus vannamei* located in north-western Mexico (Rebolledo et al., 2018). Since then, we keep this rotifer species under laboratory conditions. A monoclonal culture of the test rotifer was established using 15‰ of artificial seawater (Instant Ocean, Aquarium Systems, Inc.) at 25 ± 1 °C. Rotifers were fed with single-cell marine algae *Nannochloropsis oculata* at 3×10^6 cells mL⁻¹. Several egg-bearing individuals were separated in a 20 mL medium to obtain neonates (<8 h-old) for each experiment. *Proales similis* is a euryhaline rotifer suitable for ecotoxicological tests in different salinity scenarios ranging from 5 to 35‰ (Rebolledo et al., 2021). For all bioassays, a salinity of 15‰ was used as representative of inland sections of estuarine and lagoon ecosystems, where significant levels of pharmaceuticals and heavy metals could be regularly discharged in municipal effluents.

Chemicals

Copper and CQ stock solutions were prepared by dissolving an appropriate quantity of CuSO₄·5H₂O (J.T. Baker, >99%) and N⁴-(7-Chloro-4-quinoliny)-N¹,N¹-dimethyl-1,4-pentanediamine diphosphate salt (CAS 50-63-5 from Sigma-Aldrich, >98.5%), respectively, in milli-Q water to obtain a stock solution of 1 mg mL⁻¹.

Acute toxicity test (CQ)

Initially, we performed range-finding tests of CQ concentrations for acute toxicity testing in *P. similis*. Subsequently, six nominal concentrations of CQ (0.5, 2.5, 5.0, 7.5, 12.5 and 20.0 mg/L) plus one negative control (0) were used to evaluate the 24-h LC₅₀. Toxicity tests were conducted in borosilicate glass vials

containing 3 mL of medium (28 vials = 6 concentrations + 1 control × 4 replicates). Ten neonates were introduced into each vial. We provided a low food density (2.5×10^4 cells mL⁻¹ of *N. oculata*) during the tests in order to avoid mortality above 10%, as noted when unfed. All tests were incubated in the dark at 25 ± 1 °C for 24h. After exposure, the LC₅₀ values of CQ and their 95% confidence limits were calculated using the Probit method (Finney 1971).

Reproductive assay

According to Hernández-Flores and Rico-Martínez (2006), we measured the single and combined toxicity of CQ and Cu on the rotifer *P. similis* through chronic toxicity reproductive 5-day tests (static non-renewal testing solution). Single chronic toxicity tests were performed using an application factor (AF) of 0.05, 0.1, 0.3 and 0.5 by multiplying the 24-h LC₅₀ values of CQ (4250 µg/L) and Cu (68 µg/L (Rebolledo et al., 2021)). The combined toxicity of Cu and CQ was evaluated through five mixtures using 0.05, 0.1, 0.3 and 0.5 as AF (see Table 1). Briefly, nine neonates were introduced into a 3 mL medium with respective single and combined concentrations of CQ and Cu. Rotifers were fed *N. oculata* at a density of 1.25×10^6 cells mL⁻¹ for five days. Each treatment had one negative control group and five replicates. All bioassays were incubated in the dark at 25 °C for five days. After exposure, the final population densities of the rotifers were counted under a stereomicroscope. Population growth rate (r , d⁻¹) was calculated using the formula $r = (\ln N_t - \ln N_0) / T$, where N_t is density at time T , N_0 is density at time 0, and T is 5 days.

The concentration addition model (Broderius et al., 2005) was utilized to assess potential mixture effects of CQ and Cu. Additive effects were obtained from the sum of dividing the growth rate (r , d⁻¹) value from the CQ-Cu mixture by the corresponding r , d⁻¹ value from the sum of the toxicity of the individual chemicals. If the result was < 0.8, the action represents potential synergism (more than additive); if it was $0.8 < > 1.2$, an additive action is indicated (concentration addition); and if it was > 1.2, the action indicates potential antagonism (less than additive).

Statistical analysis

One-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test was used to compare the statistical differences between group means, where $P < 0.05$ was considered significant. All data are expressed as mean $\pm$ standard errors (SE) based on five replicate recordings.

Results

The 24-h LC₅₀ value of CQ for *P. similis* was 4.25 mg/L, with a 95% confidence interval of 3.0 to 5.9 mg/L. In general, growth rates (r , d⁻¹) of *P. similis* varied from 0.50 ± 0.02 to 0.52 ± 0.01 in the control groups. At chronic CQ exposure, ranged from 0.19 ± 0.01 to 0.39 ± 0.01 d⁻¹ (Fig. 1, M1). An increase in CQ concentration from 212 (0.05 AF) to 2125 µg/L (0.5 AF) reduced the growth rates up to 60% with respect to the controls. Regarding chronic Cu toxicity, growth rates decreased from 0.42 ± 0.01 to 0.09 ± 0.02 d⁻¹, as metal concentration increased in the medium (Fig.1, M1). At 20 (0.3 AF) and 34 (0.5 AF) µg Cu/L,

toxicity reduced growth by 58 (0.21 ± 0.01) and 82% (0.09 ± 0.02), respectively, as compared to the controls (0.52 ± 0.01).

Single and mixture toxicity of CQ and Cu to *P. similis* is shown in Figure 1. Under the M1 mixture, growth rates decreased from 0.29 ± 0.01 to $-0.14 \pm 0.03 \text{ d}^{-1}$ as AF increased in the medium. The CQ and Cu mixture effects versus single CQ and Cu exposure showed a synergistic response (Table 2). For example, at a single exposure of CQ at low AF (0.05), the r value was $0.39 \pm 0.01 \text{ d}^{-1}$. Conversely, under the CQ-Cu mixture at 0.05 AF was $0.29 \pm 0.01 \text{ d}^{-1}$, that is a 25% reduction. A similar response was observed when comparing the single toxicity of Cu versus the CQ-Cu mixture at 0.05 AF (30% reduction). At single exposure of CQ and Cu at 0.3 AF, growth rates were 0.19 ± 0.01 and $0.21 \pm 0.01 \text{ d}^{-1}$, respectively. In contrast, under the CQ-Cu mixture at 0.3 AF, it was $0.09 \pm 0.02 \text{ d}^{-1}$, reducing over 50%. The most evident synergistic effect was observed when the rotifers were exposed to a CQ-Cu mixture at 0.5 AF (Fig. 1, M1; Table 2). One-way ANOVA shows significant differences ($P < 0.05$) when comparing the individual toxicity of each chemical against the mixture at the same AF.

Regarding the M2 mixture, low AF (0.05) of CQ (213 $\mu\text{g/L}$) combined with Cu decreased growth rates from 0.29 ± 0.1 to $-0.03 \pm 0.01 \text{ d}^{-1}$ as the AF for Cu increases in the medium (Fig. 1, M2). Under these mixtures, growth rates decreased significantly ($P < 0.05$) with respect to the individual toxicity of CQ at 0.05 AF and Cu at 0.1 and 0.5 AF. We did not observe significant differences ($P > 0.05$) among single exposure to Cu at 0.3 AF and the mixture based on 0.05 AF of CQ + 0.3 AF of Cu (Fig. 1, M2). Synergistic effects were detected when combined 213 $\mu\text{g CQ/L}$ (0.05 AF) with 0.1, 0.3 and 0.5 AF of Cu (Table 2). For Cu, two synergistic and one additive effect were detected (Table 2).

The M3 mixture (Fig. 1) evidenced that all combinations of CQ at 0.1 AF (425 $\mu\text{g L}$) + Cu at 0.05, 0.3 and 0.5 AF produced a significant ($P < 0.05$) decrease in the growth rates when compared to single CQ and Cu exposure. A mixture of CQ at 0.1 AF + Cu at 0.3 AF resulted in synergistic effects; 62 and 42% reduction in growth rates observed in the individual toxicity of CQ at 0.1 AF and Cu at 0.3 AF, respectively. Using a mixture of CQ at 0.1 AF + Cu at 0.5 AF, a negative r value was observed (-0.05 d^{-1}); this was not observed in a single CQ and Cu toxicity at the same AF (Fig. 1; M3). A mixture based on CQ at 0.1 AF + Cu at 0.05 AF results in an additive effect for CQ and Cu at the same AF (Table 2). Synergistic effects were detected as Cu concentrations increased in the M3.

The M4 mixture indicated that growth rates were 22% higher when *P. similis* was exposed to CQ at 0.3 AF (1275 $\mu\text{g/L}$) + Cu at 0.05 AF, compared to single CQ exposure at 0.3 AF (Fig. 1; M4), but a one-way ANOVA showed no significant differences ($P > 0.05$) among the individual CQ exposure at 0.3 AF and the mixtures of 0.3+0.05 AF and 0.3+0.1 AF (CQ-Cu). Under a mixture of CQ at 0.3 AF + Cu at 0.5 AF, a negative r value was produced (-0.04 d^{-1}); whilst a single exposure, the r value was 0.19 d^{-1} at 0.3 AF of CQ and 0.10 d^{-1} at 0.5 AF of Cu. Combinations of CQ at 0.3 AF + Cu at 0.05 AF and CQ at 0.3 + Cu at 0.1 AF caused a significant ($P < 0.05$) decrease of 40 to 50% in growth rates compared to single Cu exposure at the same AF. An antagonism effect was detected at 0.3 AF of CQ + 0.05 AF of Cu. In the case of Cu,

this mixture resulted in an additive effect (Table 2). Synergistic effects were observed as the concentrations of CQ and Cu increased in the medium.

Regarding the chronic effect of the M5 mixture, combinations of CQ at 0.5 AF (2125 µg/L) + Cu at 0.1 and 0.3 AF decreased significantly ($P < 0.05$) growth rates when compared to single chemical exposure (Fig. 1; M5). A mixture of CQ at 0.5 AF + Cu at 0.05 (3.5 µg/L) did not differ from the individual CQ toxicity at 0.5 AF. However, it caused a 50% reduction in growth rates than single Cu exposure at the same AF. A mixture of CQ at 0.5 AF + Cu at 0.3 AF (20 µg/L) resulted in a low r value (0.05 d^{-1}). A more noticeable synergistic response was observed under a mixture of CQ at 0.5 AF + Cu at 0.3 AF; growth rates decreased more than 75% than single Cu and CQ exposure at the same AF. Two additive effects were found for CQ and one synergistic response at 0.5 AF of CQ + 0.3 AF of Cu. For Cu, all combinations induced synergistic effects (Table 2).

Discussion

In the present study, we examined the toxicity of chloroquine due to its current and future potential applications to treat several human diseases. Its important use and high potential to being persistent make it of particular concern for ecotoxicological studies. Presumably, as has occurred with other pharmaceuticals drugs, CQ will gain more ecotoxicological relevance after this worldwide crisis (Essid et al., 2020; Ali et al., 2021; Kuroda et al., 2021).

Hitherto, limited information is available regarding the acute and chronic toxicity of CQ in aquatic biota. As a first approximation of CQ toxicity, the euryhaline rotifer *P. similis* has a 24-h LC_{50} of 4.25 mg/L. This result is very close to that for the freshwater rotifer *Brachionus calyciflorus* (4.39 mg/L) and the anostracan *Thamnocephalus platyurus* (4.70 mg/L), organisms widely used in ecotoxicological studies (Calleja et al., 1994; Nalecz-Jawecki and Persoone, 2006). *Proales similis* seems to be more sensitive to CQ than the cladoceran *Daphnia magna* (24-h EC_{50} , 21.5 to 43 mg/L) and the crustacean *Streptocephalus proboscideus* (24-h EC_{50} , 11.6 mg/L) (Calleja et al., 1994; Zurita et al., 2005). It is documented that some vertebrates, such as *Cyprinus carpio*, have a 96-h LC_{50} of 31.6 CQ mg/mL (Ramesh et al., 2018) that far exceeds the tolerance of the previously mentioned invertebrates. Considering that the sensitivity of *P. similis* to CQ is within the range reported in standard test species for ecotoxicological assays, we suggest that this marine rotifer be used as an indicator for ecological risk assessment of emerging pollutants that currently deserve more attention.

The chronic toxicity of CQ has been rarely examined in aquatic organisms (Ramesh et al., 2018). As many as 10 µg CQ/L have been reported in the field, mainly near hospital areas (Olatunde et al., 2014). It is predicted that this concentration in aquatic ecosystems will increase due to the COVID-19 pandemic (Espejo et al., 2020; Kuroda et al., 2021). In our present study, the chronic test concentrations were based on the LC_{50} value of CQ for *P. similis*. These concentrations have not yet been detected in the field. However, they can be predictive not only for this rotifer species but also for other zooplankton species with a similar response to CQ. Obtained data indicate that from 213 to 2125 µg CQ/L (5 to 50% of LC_{50}),

the growth rates of *P. similis* are significantly reduced (22 to 60%). A similar amount (200 µg/L) of other pharmaceuticals, such as amoxicillin, cause significant effects on the rate of population increase (60% of reduction) on *Brachionus havanaensis* (González-Pérez et al., 2016). Unlike *P. similis*, the growth of some aquatic organisms is inhibited at pharmaceutical concentrations ranging from 5.0 to 300 mg/L (Watanabe et al., 2015; Godoy et al., 2019), or even as much as 2000 mg/L (Magdaleno et al., 2015). Recently, Ali et al. (2021) found that chronic exposure to high hydroxychloroquine (a less toxic derivative of CQ) concentrations (31.62 and 63.24 µg/mL) impact the abundance and diversity of marine nematodes negatively. Therefore, the present data highlights the toxic potential of CQ at moderate concentrations for marine zooplankton populations.

Copper toxicity is known in a wide diversity of aquatic organisms, including zooplankton (Arnold et al., 2011). Our results agree with those of Rebolledo et al. (2021); *P. similis* is more sensitive to Cu than other saline or marine rotifers. For example, at 34 µg Cu/L (50% of the LC₅₀), growth rates of *P. similis* are reduced by as much as 83%. At the same time, populations of *B. rotundiformis* can remain unaffected at a chronic exposure of 31 to 125 µg CuSO₄/L (Gama-Flores et al., 2005). Conversely, it is suggested that *B. plicatilis* is even more resistant since it can grow under a chronic concentration of 500 µg CuSO₄/L that exceeds the LC₅₀ of *P. similis* (Luna-Andrade et al., 2002; Rebolledo et al., 2021). Snell et al. (2019) found that some complex strains of *B. plicatilis* might be more sensitive to Cu compared to *P. similis*, therefore suggests that their sensitivity is not a generality compared to other marine rotifers. Cu concentrations used in our chronic tests (3.5 to 34 µg/L) are below some levels detected (ca. 50 µg/L) in estuaries and coastal marine environments (e.g., Vazquez et al., 1999; Jonathan et al., 2011). It is assumed that the demographic parameters of *P. similis*, as well as other zooplankton taxa with high sensitivity to Cu, are negatively affected by chronic metal exposure that exceeds the tolerance threshold of this species (Bechmann 1994; Schuler et al., 2008; Kwok et al., 2008; Arnold et al., 2011).

Usually, the toxicities of pollutants mixtures are reported more as additive than synergistic effects (Cedergreen 2014), which has led regulatory authorities to consider that the toxicity of a combination of chemicals will be approximately additive (Walker et al., 2012). To our knowledge, this is the first study to demonstrate the mixture toxicity of CQ and Cu in marine invertebrates. The present findings underline the risk of the different mixtures of these two chemicals; in most cases, they induced a synergistic effect on the population growth rate of *P. similis*. Contrary to Almeida et al. (2018), who found that a mixture of drugs (carbamazepine and cetirizine) with a heavy metal (Cd) had lower biological effects than the contaminants alone in the clam *Ruditapes philippinarum* and that the metal attenuated the drugs effects; in this study, the combined toxicity of CQ with a heavy metal (Cu) exacerbated the vulnerability of the organisms to the drug, as reported by Ji et al. (2020). It is not the first case that the combination of Cu with other pollutants induces synergistic effects on the growth and survival of marine invertebrates (Bao et al., 2013; 2014). A synergistic effect of the CQ-Cu mixture will have occurred when these chemicals have been combined at high concentrations. Tested concentrations of Cu are within those detected in marine environments (Jonathan et al., 2011), but in the case of CQ, these are well below environmental concentrations (Olatunde et al., 2014). Thus, their importance as synergists within naturally occurring

realistic scenarios is a relatively minor concern (Cedergreen 2014). Nevertheless, this aspect should not be understated, given the current environmental threats.

Copper has been recognized as one most toxic heavy metals to aquatic invertebrates. In particular, Cu is more toxic than Fe, Pb, Cd, Zn, As and Cr to *P. similis* (Rebolledo et al., 2021). In this work, it was noticed that growth rates were more affected by Cu at high concentrations than by CQ. When these chemicals were combined, we assumed that Cu exerted more pressure than CQ, thus intensified the sensitivity of organisms to the tested drug. Therefore, the widespread contamination of Cu in coastal environments and its high toxicity to aquatic life should be considered more frequently for the environmental risk assessment of chemical mixtures. It is essential to mention that the toxicity of mixtures of CQ and Cu to *P. similis* could increase under ecological stress (Shahid et al., 2019; Rebolledo et al., 2021). Future research should examine this aspect.

Conclusion

According to the literature, the sensitivity of *P. similis* to CQ found in the present study is within the range reported in standard test species for ecotoxicological testing. We suggest that this rotifer species serve as a test organism to investigate the risk assessment of emerging pollutants in marine environments. In general, a low concentration exposure of each chemical tested (CQ and Cu) was enough to exert significant effects on the population growth of rotifers; these were intensified when the compounds acted in combination. CQ-Cu mixtures exhibited a strong synergistic effect on *Proales* mainly as the concentrations of each chemical increased in the medium. Synergistic response stresses the potential hazard that these combined chemicals may have on the aquatic systems and evidence of the possible environmental risks associated with emerging pollutants that have originated during the COVID-19 pandemic. At present, we must show the effects of global pollution from a more realistic perspective, where pollution by a single chemical is rare or does not exist.

Declarations

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Author contributions All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Consent to participate All authors consent to participate.

Consent for publication All authors consent for publication.

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Tables

Table 1. Nominal concentrations ($\mu\text{g L}^{-1}$) of chloroquine (CQ) and copper (Cu) according to the different application factors (AF). Five combinations of CQ and Cu based on the different AF are shown.

Treatment	AF (CQ + Cu)	Concentration ($\mu\text{g/L}$)	
		CQ	Cu
M1	0.05 + 0.05	213	3.5
	0.1 + 0.1	425	6.8
	0.3 + 0.3	1275	20
	0.5 + 0.5	2125	34
M2	0.05 + 0.1	213	6.8
	0.05 + 0.3	213	20
	0.05 + 0.5	213	34
M3	0.1 + 0.05	425	3.5
	0.1 + 0.3	425	20
	0.1 + 0.5	425	34
M4	0.3 + 0.05	1275	3.5
	0.3 + 0.1	1275	6.8
	0.3 + 0.5	1275	34
M5	0.5 + 0.05	2125	3.5
	0.5 + 0.1	2125	6.8
	0.5 + 0.3	2125	20

Table 2. Effects of CQ-Cu mixtures on the growth rates of *Proales similis* at different application factors (AF) for each mixture. < 0.8 indicates synergy, $0.8 < > 1.2$ additive, > 1.2 antagonism.

		Cu			
CQ	AF	0.05	0.1	0.3	0.5
	0.05	0.74	0.75	0.20	-0.71
	0.1	0.84	0.67	0.39	-0.18
	0.3	1.29	0.87	0.49	-0.23
	0.5	1.05	0.82	0.20	-0.71

		CQ			
Cu	AF	0.05	0.1	0.3	0.5
	0.05	0.68	0.64	0.59	0.50
	0.1	0.86	0.63	0.49	0.49
	0.3	1.05	0.60	0.45	0.20
	0.5	-0.33	-0.64	-0.48	-1.58

Figures

Figure 1

Growth rates of *Proaetes similis* exposed at single and mixture (M1 – M5) toxicity of chloroquine (CQ) and copper (Cu) under different application factors (AF). Distinct letters indicate significant ($P < 0.05$) differences among individual chemical toxicity and combined toxicity of CQ and Cu.